

AWARD NUMBER: W81XWH-16-1-0531

TITLE: Epigenetic machinery regulates alternative splicing of androgen receptor (AR) gene in castration-resistant prostate cancer

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REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE September 2017	2. REPORT TYPE Annual	3. DATES COVERED 09/01/2016-08/31/2017		
4. TITLE AND SUBTITLE Epigenetic machinery regulates alternative splicing of androgen receptor gene in castration-resistant prostate cancer			5a. CONTRACT NUMBER	
			5b. GRANT NUMBER W81XWH-16-1-0531	
			5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Zhi-Ping Liu E-Mail: zhi-ping.liu@utsouthwestern.edu			5d. PROJECT NUMBER	
			5e. TASK NUMBER	
			5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UT Southwestern Medical Center 5323 Harry Hines Blvd., Dallas, TX 75390			8. PERFORMING ORGANIZATION REPORT NUMBER	
			10. SPONSOR/MONITOR'S ACRONYM(S)	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
			12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited	
13. SUPPLEMENTARY NOTES				
14. ABSTRACT Androgen deprivation therapy (ADT) is the primary treatment for metastatic prostate cancer (PCa) since PCa depends on androgen for growth. Although initially responsive, most tumors progress into androgen-independent/castration-resistant PCa (CRPC). No curative therapy is available. One of the reasons for the resistance to ADT and newer anti-androgen drugs is the emergence of constitutively active AR variants (AR-Vs) such as AR-V7 that are induced under ADT conditions. Our research goal is to test the hypothesis that the epigenetic regulator KDM4B, a histone lysine demethylase, promotes AR-V7 via alternative splicing, leading to CRPC. A multi-disciplinary approach including molecular biology, tumor biology, cell biology, and biochemical method is used to test this hypothesis. In collaboration with a partnering principal investigator we are also testing the efficacy of our newly identified KDM4B inhibitor(s) as a monotherapy or combined with approved anti-androgen agents in AR-V7-expressing CRPC in preclinical mouse models.				
15. SUBJECT TERMS Nothing listed				
16. SECURITY CLASSIFICATION OF: a. REPORT Unclassified		17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 13	19a. NAME OF RESPONSIBLE PERSON USAMRMC
				19b. TELEPHONE NUMBER (include area code)
b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	5
5. Changes/Problems	6
6. Products	6
7. Participants & Other Collaborating Organizations	7
8. Special Reporting Requirements	7
9. Appendices	8

1. INTRODUCTION:

The subject of our research is to identify the molecular mechanism of the drug resistance in castration-resistant prostate cancer (CRPC). Our preliminary data suggest that one of the mechanisms of the resistance is the emergence of constitutively active androgen-receptor variants such as AR-V7. Our goals are to demonstrate that histone lysine demethylase KDM4B regulates AR-V7 via alternative splicing and to test the efficacy of our newly identified KDM4B inhibitor(s) as a monotherapy or combined with approved anti-androgen agents in AR-V7-expressing CRPC in pre-clinical animal models of CRPC.

2. KEYWORDS:

Histone lysine demethylase, castration-resistant prostate cancer, alternative splicing, AR-V7, KDM4B, small molecule inhibitors.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

There are two specific aims in this proposal. We have made significant progresses for both aims in the past year. One manuscript for publication is written and is currently under review.

Aim 1. To establish that KDM4B promotes AR-V7 expression and identify the regulatory mechanisms.

Major Task 1: Determine the role of KDM4B in promoting AR-V7 expression in various PCa cell lines, including those resistant to enzalutamide.—**completed (6/30/2017).**

Major Task 2: Determine how KDM4B binds to the spliceosome associated with pre-mRNA.—**completed (6/30/2017).**

Milestone #1: Co-author manuscript on KDM4B-RNA interaction.—We have met this milestone. The manuscript was submitted to cancer cell and is currently under review (6/30/2017).

Major Task 3: Map RNAPII, H3K9/K36me3 occupancy around AR locus using ChIP-qPCR in several CRPC cells.—in progress, 30% completed.

Major Task 4: To identify potential KDM4B-regulated alternative splice gene(s) using RNA-seq and map KDM4B-RNA interactions with CLIP-seq.—in progress, 30% completed.

Aim 2. To evaluate the clinical application of KDM4B inhibitors on CRPC tumors expressing AR-Vs.

Major Task 5: Identify two lead compounds using CRPC cell lines and optimizing their dosage and schedule in xenograft models.—in progress, 30 completed. please see the progress report from the partner of the project Dr. JT Hsieh (PC150152P1) for details.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) major activities;

- a) Measured the expression of KDM4B and AR-V7 in various PCa cell lines, tested whether their expression can be upregulated under ADT conditions.
- b) Knockdown KDM4B using KDM4B specific siRNAs.
- c) Employed CRISPR/Cas9 technology to permanently delete KDM4B in PCa cell lines.

- d) Characterized KDM4B-null cells and compared them with parent cell line using MTT, Transwell, soft agar assays, and xenografts.
- e) Performed RIP and CLIP-qPCR assays to test whether KDM4B binds RNA and identify RNA binding sites
- f) Performed RNA-gel shift assays to characterize KDM4B-RNA interaction.

2) Specific objective;

We completed part of the specific aim 1. We established that KDM4B promotes AR-V7 expression and identified several the regulatory mechanisms.

3) Significant results or key outcomes;

Significant results. We found that KDM4B is necessary and sufficient to promote AR-V7 expression in PCa cell lines (please see Fig. 1 in appendix). While overexpression of KDM4B promoted PCa cell growth and migration, and tumor take-up rate (Fig. 2), knockdown (KD) of *KDM4B* inhibited tumor growth in castrated mice and abolished tumor AR-V7 expression (Fig 3), suggesting that KDM4B promotes CRPC, at least partially via AR-V7. Pharmacological inhibition of CRPC xenograft with the KDM4 inhibitor B3 suppressed tumor growth, diminished AR-V7 expression and sensitized CRPC cells to enzalutamide inhibition (Fig. 4). KDM4B expression significantly correlates with that of AR-V7 in PCa patients (Fig. 1G), and higher KDM4B expression predicts poor prognosis (Fig. 2). Mechanistically, KDM4B can interact with components of the spliceosome (Fig. 5). KDM4B binds directly to the pre-mRNA near 3'ss of CE3 and promotes alternative splicing of AR-V7 (Fig. 6). These data suggest that KDM4B may promote usage of the 3'ss of CE3 via enhancing/stabilizing the recruitment of the spliceosome.

Key outcomes. Our studies demonstrate that KDM4B is a bona fide splicing regulator, which could be a cancer-specific alternative-splicing regulator that dictates oncogenic alternative splicing. Targeting KDM4B could inhibit CRPCs that are refractive to current androgen-deprivation therapy.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

We have submitted a manuscript for publication

What do you plan to do during the next reporting period to accomplish the goals?

We are on target following the plan stated in the SOW. For the next reporting period, we will continue to map RNAPII, H3K9/K36me3 occupancy around AR locus using ChIP-qPCR in several CRPC cells (major task 3) and to identify potential KDM4B-regulated alternative splice gene(s) using RNA-seq and map KDM4B-RNA interactions with CLIP-seq (major task 4).

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project? What was the impact on other disciplines?

Alternative splicing is emerging as an important determinant of oncogenesis, response to treatment, and drug resistance, thus representing an important vulnerability with potential to be exploited for therapeutic purposes. Epigenetic changes including histone modifications have long been recognized to play a role in tumor initiation and progression. Here, we identified a novel mechanistic link between alternative splicing and histone lysine demethylase KDM4B. KDM4B is overexpressed in many human cancers including prostate cancer. We show that KDM4B functions as both a chromatin remodeler and a trans-acting splicing factor, brings the splicing machinery to the chromatin. We identified many KDM4B-targeted genes including AR whose alternatively spliced isoforms have been implicated in general tumorigenesis, thus demonstrating the far-reaching significance of this novel mechanism.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to report

- 5. CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

- 6. PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
Nothing to report
- **Books or other non-periodical, one-time publications.**
Nothing to report
- **Other publications, conference papers and presentations.**
Nothing to report
- **Website(s) or other Internet site(s)**
Nothing to report
- **Technologies or techniques**
Nothing to report
- **Inventions, patent applications, and/or licenses**
Nothing to report
- **Other Products**
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: *Zhi-Ping Liu*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): *0000-0003-1341-3878*
Nearest person month worked: *3*
Contribution to Project: *Designed experiments, analyzed data, write progress report, and manuscript.*
Funding Support: *Cancer prevention and research institute of Texas (CPRIT), American heart association (AHA), DOD, NIH*

Name: *LingLing Duan*
Project Role: *Research associate*
Researcher Identifier (e.g. ORCID ID): *none*
Nearest person month worked: *8*
Contribution to Project: *designed and performed experiments, analyzed data, write progress report, and manuscript.*
Funding Support: *Cancer prevention and research institute of Texas (CPRIT)*

Name: *Qing-Jun Zhang*
Project Role: *Research Associate*
Researcher Identifier (e.g. ORCID ID): *none*
Nearest person month worked: *4*
Contribution to Project: *performed experiments and analyzed data*
Funding Support: *Cancer prevention and research institute of Texas (CPRIT), American heart association (AHA), DOD, NIH*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: please see Partnering PI Jer-Tsong Hsieh's progress report

QUAD CHARTS: If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

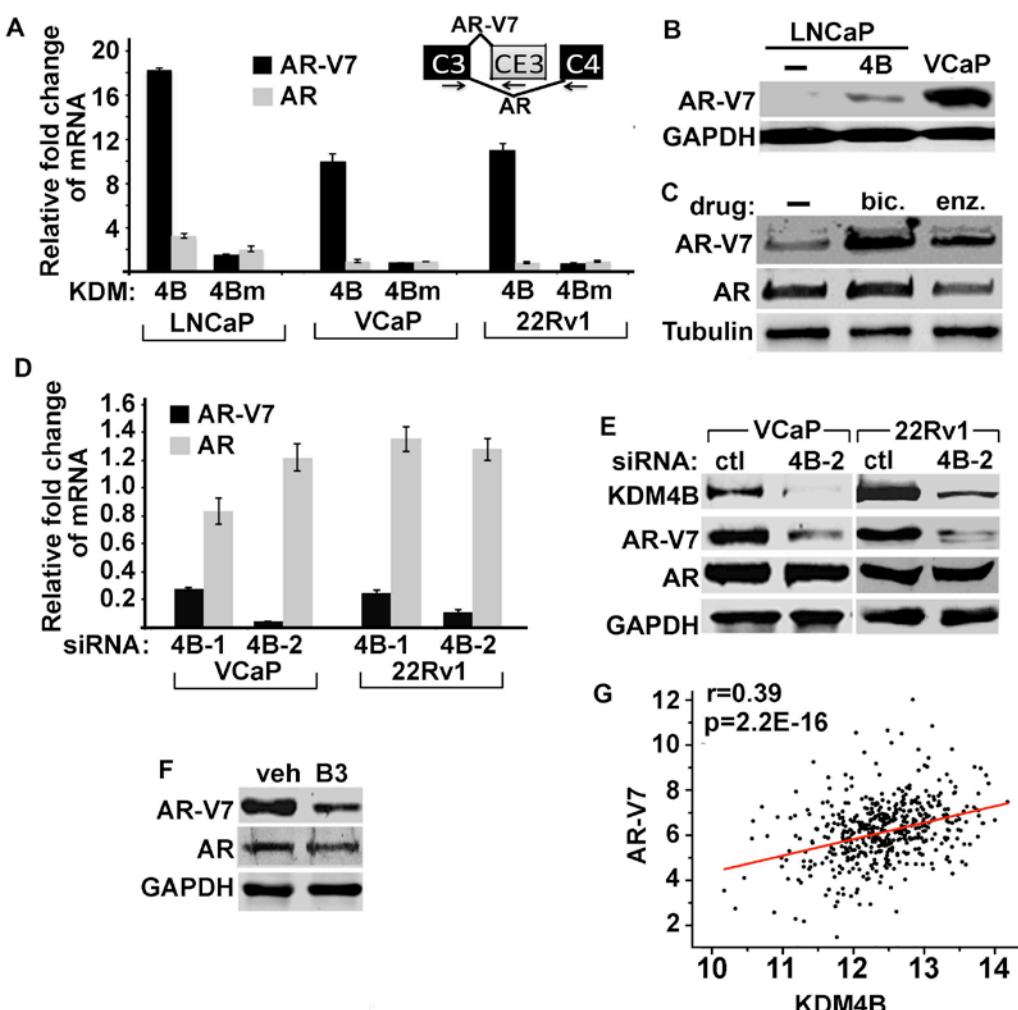


Figure 1. KDM4B promotes AR-V7 expression. (A) Relative fold change of mRNAs of AR-V7 and AR in LNCaP, VCaP and 22Rv1 cells transfected with KDM4B (4B) or mutant KDM4B^{H188A} (4Bm) expression plasmid. mRNA was normalized against internal Calnexin and expressed relative to that of vector transfected cells ($n=3$, mean \pm SEM). (B) Western blot of AR-V7 protein in LNCaP, LNCaP-4B, and VCaP cells. GAPDH was used as loading control. (C) Western blot of AR-V7 and AR in LNCaP-4B cells cultured in charcoal-stripped FBS (CFBS) in the absence or presence of anti-androgen bicalutamide (bic, 10 μ M) or enzalutamide (enz, 10 μ M). (D) The mRNA and (E) protein levels of AR-V7 and AR in VCaP and 22Rv1 cells transfected with control (ctl) or KDM4B siRNA. mRNA was normalized against internal Calnexin and expressed relative to that of control siRNA-transfected cells. (F) Western blot of AR-V7 and AR in 22Rv1 cells treated with vehicle (veh) or KDM4B inhibitor B3. GAPDH was used as loading control. (G) Pearson's correlation coefficient analysis between mRNAs of KDM4B and AR-V7 from the TCGA database.

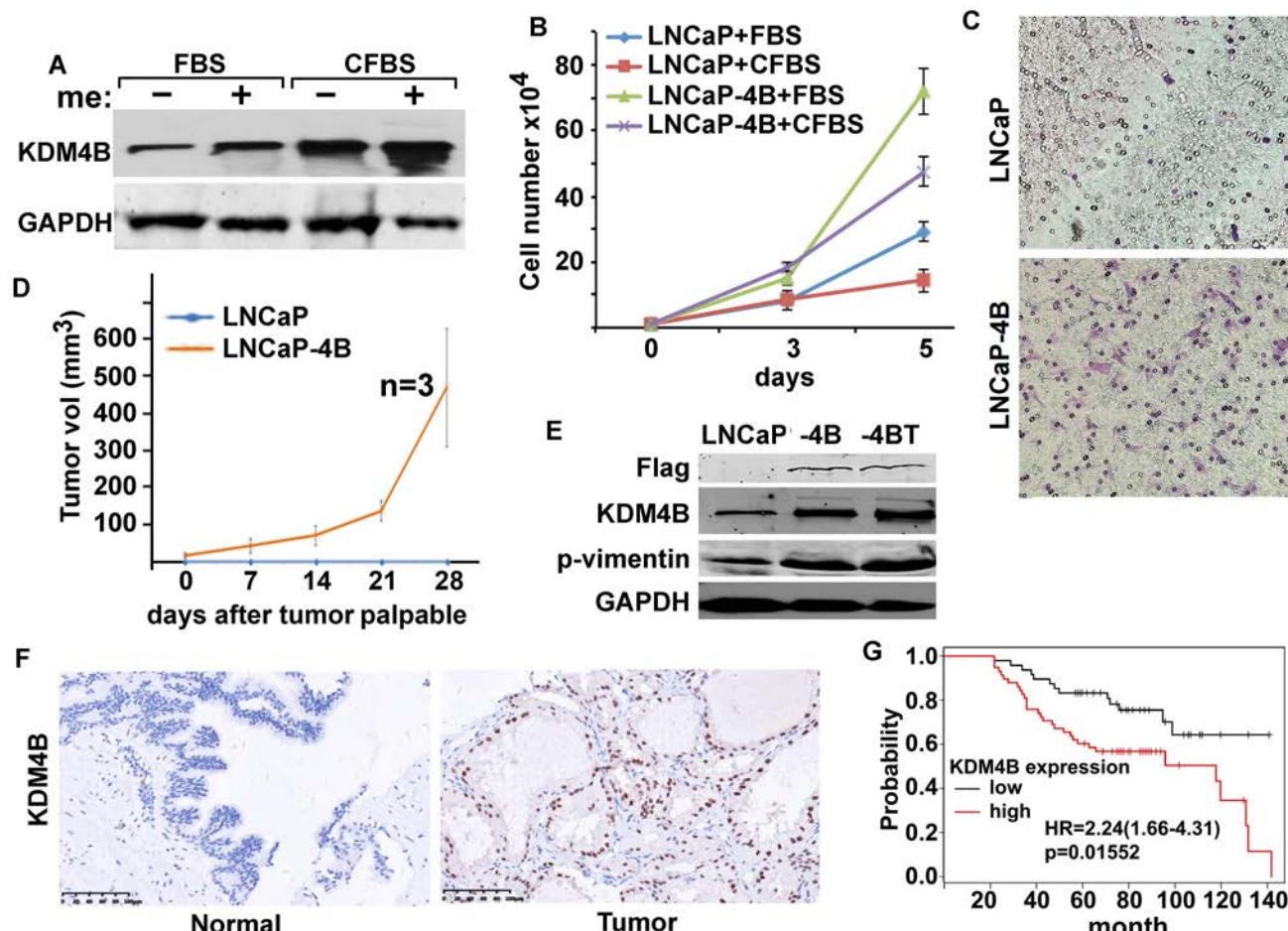


Figure 2. KDM4B promotes prostate tumorigenicity. (A) KDM4B expression in LNCaP cell culture without (-) or with methylcellulose (me) in the presence of FBS or CFBS. (B) Growth curves of LNCaP and LNCaP-4B cells in FBS or CFBS. (C) Transwell assay showing that LNCaP-4B cells (stained with hematoxylin, in blue) are significantly more migratory. (D) Growth curves of xenografts derived from LNCaP-4B and LNCaP cells (n=10/group, mean± SD). 30% of tumor take-up rate was observed for LNCaP-4B xenograft. (E) Western blot of lysates from LNCaP, LNCaP-4B (4B), and cells re-plated from LNCaP-4B xenograft tumor (-4BT). (F) IHC staining of KDM4B in prostate adenocarcinoma and normal tissue. (G) Kaplan–Meier analysis of the correlation between KDM4B protein level in (F) and the overall survival of patients with metastatic PCa (n=106).

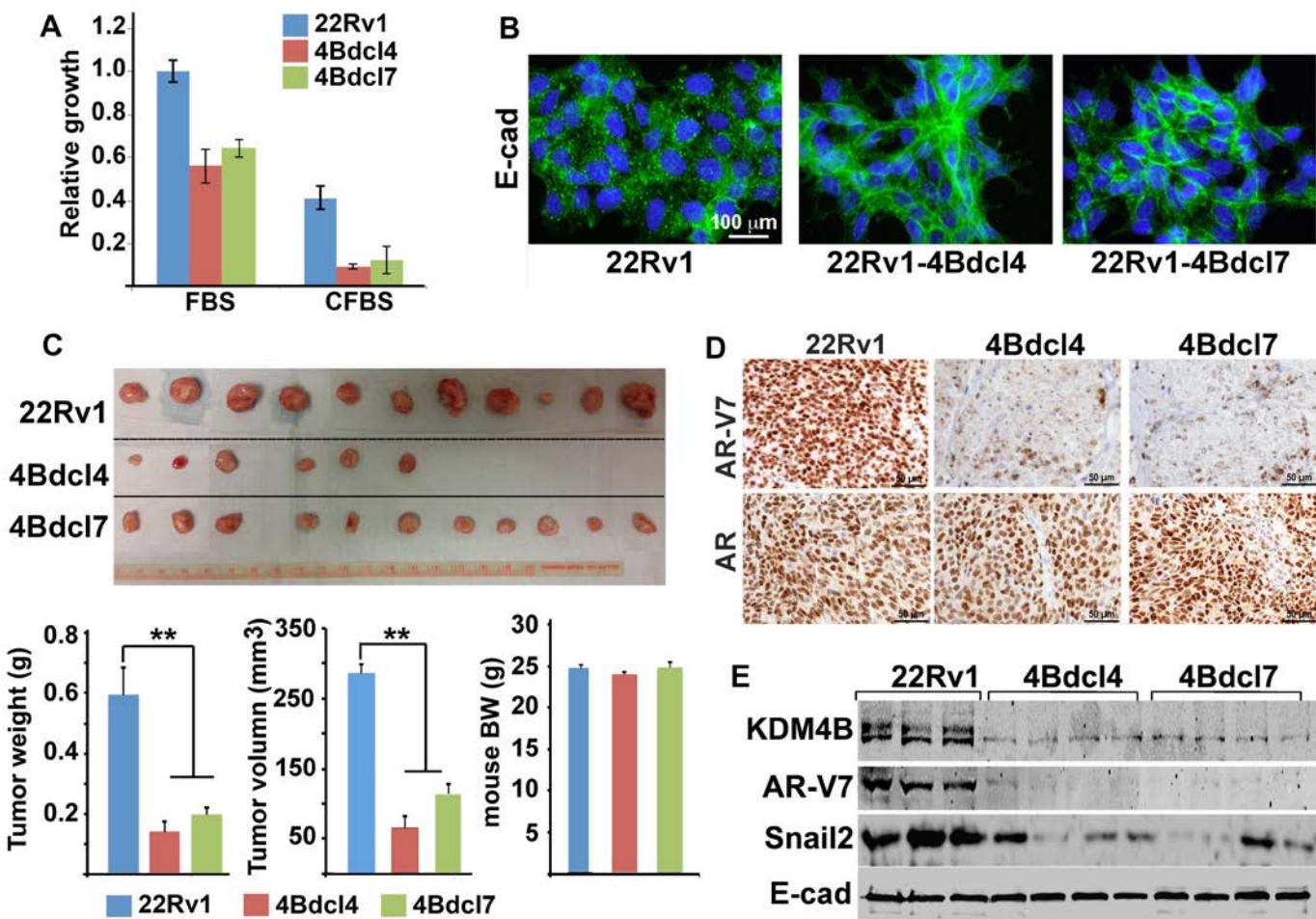


Figure 3. KDM4B knockdown inhibits CRPC growth in vivo and AR-V7 expression. (A) Relative growth of 22Rv1, and clonal 22Rv1 cells with KDM4B-heterozygously deleted (4Bdcl4 and 4Bdcl7). Equal amounts of cells were seeded in medium supplemented with FBS or CFBS. MTT assays were performed 3 days later. (B) Immunofluorescence micrographs of cells in (A) stained with E-cadherin (E-cad, green) and DAPI (blue). (C) Tumor xenografts derived from 22Rv1, 4Bdcl4, and 4Bdcl7 cells (upper panel). The lower panel shows tumor weight and volume, and castrated host mice weights at time of sacrifice. **, $p < 0.01$. (D) Representative IHC staining of AR-V7 and AR in tumors of (C). (E) Western blot of indicated proteins from tumors of (C).

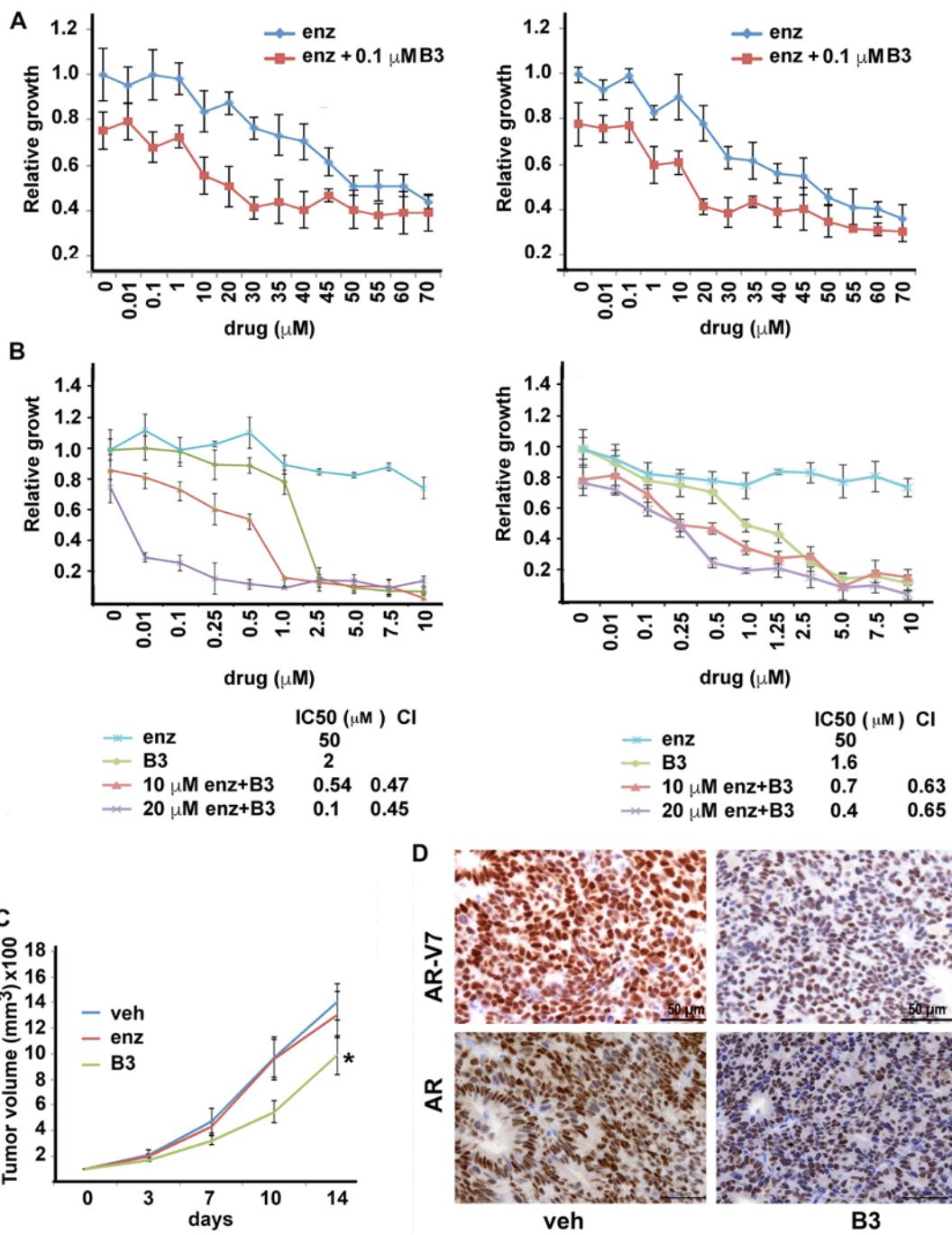


Figure 4. KDM4B inhibitor B3 sensitizes the response of CRPC cell lines to enzalutamide and inhibits castration-resistant tumor growth. (A) Growth curves of 22Rv1 (left panel) and VCaP (right panel) cells in the presence of various concentration of enzalutamide (enz) alone or in the presence of B3 (0.1 μM). (B) Dose response curves of 22Rv1 (left panel) and VCaP (right panel) cells to B3, enz, or in combination as indicated. CI was calculated based on the IC₅₀s of single compound or compounds in combination and listed below the curves. (C) Tumor growth curves of 22Rv1-xenografts treated with veh (n=6), enzalutamide (n=8), or B3 (n=11). Mean \pm SEM, *, p<0.05. (D) IHC of AR and AR-V7 in tumors from (C).

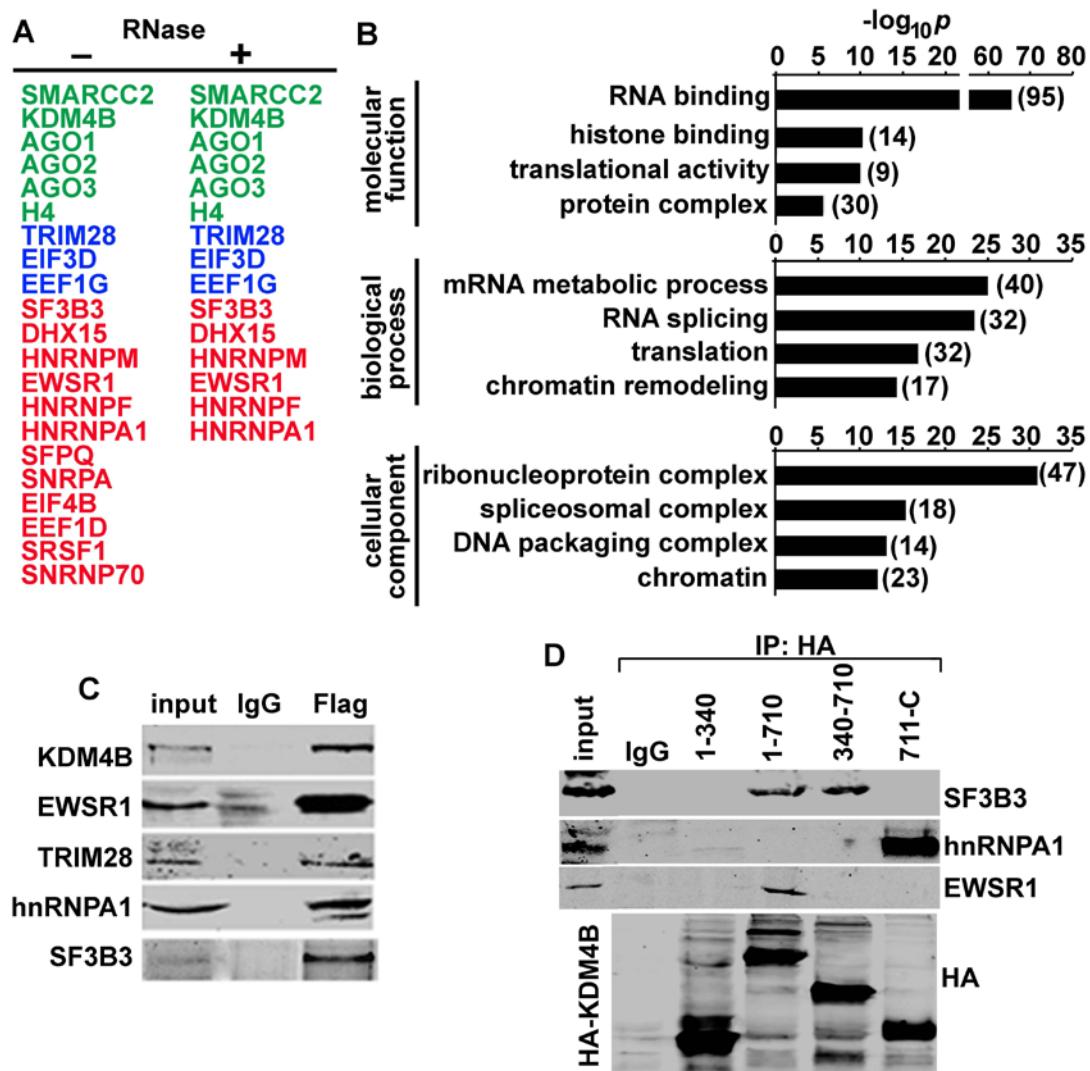
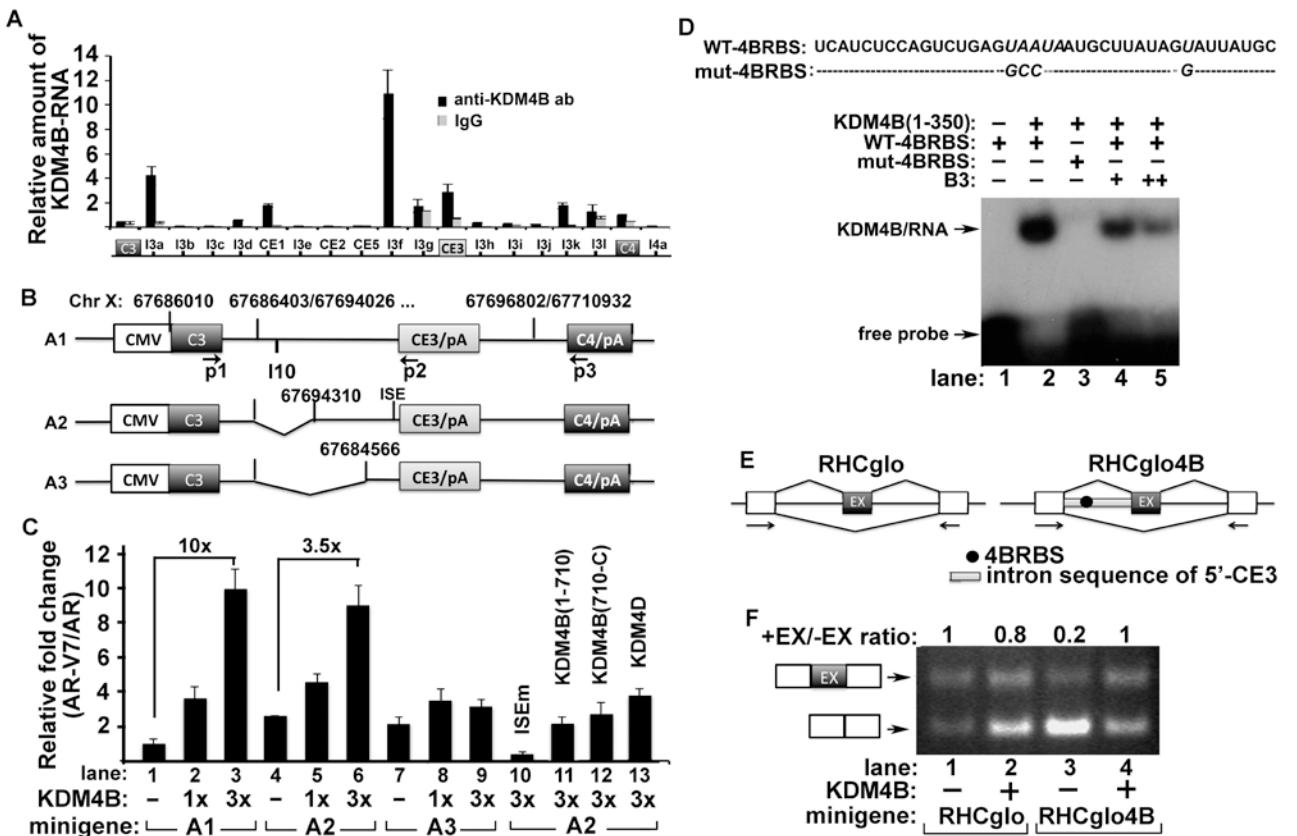


Figure 5. KDM4B is associated with splicing factors. (A) LNCaP and LNCaP-4B cells were immunoprecipitated with Flag antibody. Immunocomplexes were treated with or without RNase, eluted with 3xFlag peptide, and subjected for mass-spectrometry. Proteins that are uniquely identified in LNCaP-4B cells are listed. Components of the spliceosome are highlighted in red, chromatin modifiers in green, and proteins involved in transcription in blue. (B) GO analysis of the KDM4B-interacting proteins indicated that a majority of the proteins are involved in RNA metabolism. (C) WB of indicated proteins from immunoprecipitates of LNCaP-4B cells immunoprecipitated with IgG or anti-Flag antibody. (D) HEK293T cells were transfected with HA-tagged KDM4B plasmid expressing different regions of KDM4B. Cell lysates were immunoprecipitated with anti-HA antibody and probed with anti-HA, hnRNPA1, SF3B3 and EWSR1 antibodies.



WT-4BRBS: UCAUCUCCAGUCUGAGUAAAUGGCUUAAGUAUUAG
mut-4BRBS: GCCG

KDM4B(1-350): - + + + +
WT-4BRBS: + + - + +
mut-4BRBS: - - + - -
B3: - - - + ++

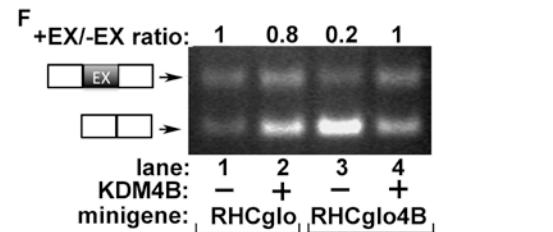
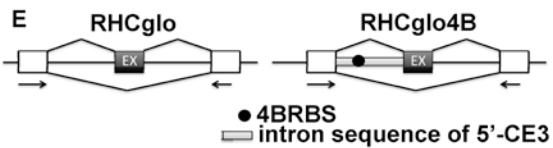
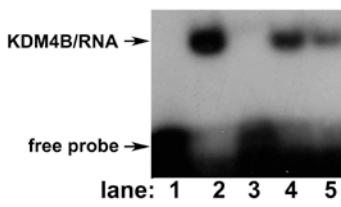


Figure 6. KDM4B promotes alternative splicing of AR-V7 as a trans-acting splicing factor. (A) RNA immunoprecipitation with anti-KDM4B antibody or control IgG. The immunoprecipitates were extracted with Trizol. The relative occupancy of KDM4B at each RNA site over the AR pre-mRNA was quantitated by qRT-PCR with primer pairs indicated below the graph. (B) Schematics of AR-V7 minigenes with various intronic sequences 5'-upstream of CE3. (C) Minigene reporters (A1, A2, or A3) (1.5 μ g) with indicated expression vectors (400ng, 1x) were transfected into HEK293 cells. RNAs were extracted 48 h post-transfection. Expression levels of AR and AR-V7 were determined by qRT-PCR using primer pair p1/p3 and p1/p2, respectively. The ratios of AR-V7 over AR were expressed relative to the A1-minigene alone ($n=3$, mean \pm SEM). (D) RNA-gel shift assay with KDM4B(1-350) and 32 P-labeled RNA probes (WT-4BRBS, mut-4BRBS) in the absence or presence of KDM4B inhibitor B3. (E) Schematic of minigene reporter RHCglo and RHCglo4B. In RHCglo4B, the intronic sequence between exons 1 and EX and 3'-splice site next to EX in RHCglo were replaced with 500 bp genomic sequence 5'-upstream of CE3 that contained 4BRBS and 3'-splice site of CE3, respectively. (F) Indicated minigene assays with or without transfected KDM4B. RNA products with EX-included (+EX) or excluded (-EX) were measured by RT-PCR using the primers shown in (E).